Dietary Intake of Ascorbic Acid Attenuates Lipopolysaccharide-Induced Sepsis and Septic Inflammation in ODS Rats

Noe KAWADE1, Yuki TOKUDA1, Shogo TSUJINO1, Hiroaki AOYAMA2, Misato KOBAYASHI1, Atsushi MURAI1 and Fumihiko HORIO1,∗

1Department of Animal Sciences, Graduate School of Biagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan
2Toxicology Division, Institute of Environmental Toxicology, Ibaraki 303–0043, Japan

Summary The aim of this study was to verify the protective effects of ascorbic acid (AsA) against lipopolysaccharide (LPS)-induced sepsis. The study was conducted using osteogenic disorder Shionogi (ODS) rats, which are unable to synthesize AsA. Male ODS rats (6 wk old) were fed either an AsA-free diet (AsA-deficient group), a diet supplemented with 300 mg/kg AsA (control group), or a diet supplemented with 3,000 mg/kg AsA (high-AsA group) for 8 d. On day 8, all the rats were intraperitoneally injected with LPS (15 mg/kg body weight). Forty-eight hours after the injection, the survival rates of the rats in the control (39%) and the high-AsA (61%) groups were significantly higher than that in the AsA-deficient group (5.5%). Next, we measured several inflammatory parameters during 10 h after administering LPS. At 6 h, elevated serum levels of markers for renal injury were suppressed in rats fed AsA. Similarly, 10 h after LPS injection, the elevation in the serum levels of markers for renal injury were also suppressed proportionally to the amount of AsA in the diet. The elevated serum concentrations of TNFα and IL-1β by LPS in the AsA-deficient group decreased in groups fed AsA. Hematoc TNFα mRNA levels at 6 h after the LPS injection were also lowered by feeding AsA. These results demonstrated that the dietary intake of AsA improved the survival rates and suppressed the inflammatory damage, in a dose-dependent manner, caused during sepsis induced by LPS in ODS rats.

Key Words ascorbic acid, lipopolysaccharide, sepsis, inflammatory damage, ODS rat

Ascorbic acid (AsA; vitamin C) is a water-soluble molecule that acts as an antioxidant and enzyme cofactor. Humans, other primates, guinea pigs and osteogenic disorder Shionogi (ODS) rats cannot synthesize AsA due to the hereditary lack of L-gulono-γ-lactone oxidase (EC 1.1.3.8), which catalyzes the terminal reaction in AsA biosynthesis (1, 2). The dietary addition of 300 mg of AsA per kg diet is sufficient for optimum growth and prevention of scurvy in ODS rats (3). We have investigated the novel physiological roles of AsA using ODS rats. We have previously demonstrated that the serum concentrations of proinflammatory cytokines, such as interleukin-6 (IL-6) and IL-1β, and acute phase proteins (APPs) were elevated in AsA-deficient ODS rats, and that AsA deficiency in ODS rats promoted the expressions of various hepatic genes encoding for APPs including C-reactive protein (CRP) and haptoglobin (4, 5). These observations in AsA-deficient ODS rats are similar to the observations during low-grade inflammation induced by bacterial infection. Moreover, previous studies done on humans also revealed the inverse correlation between serum concentrations of AsA and CRP (6–9). CRP is an APP and is considered to be a biomarker of systemic inflammation. The elevation of serum CRP levels induced by AsA deficiency in humans is similar to our observations in AsA-deficient ODS rats. These results in both ODS rats and humans suggest that AsA possesses anti-inflammatory property.

In this study, to examine the anti-inflammatory action of AsA, we attempted to verify the protective effects of AsA against the lipopolysaccharide (LPS)-induced sepsis in ODS rats. Sepsis refers to systemic inflammatory response syndrome (SIRS) caused by bacterial infection. In severe septic condition, organ dysfunction and hypotension occur, and these symptoms often prove to be fatal. Sepsis is the primary cause of death in the intensive care unit (ICU), and approximately 18 million people die of sepsis every year globally (10, 11). LPS, a component of the outer membrane of gram-negative bacteria, induces septic inflammatory responses through toll-like receptor 4 (TLR4) (12).

Previous investigations into the protective effects of AsA against sepsis mainly used mice or rats which could synthesize AsA. These reports indicated that intravenous or intraperitoneal injections of AsA improved the survival rates, suppressed the organ dysfunctions, and downregulated the expressions of inflammatory cytokine genes under septic conditions (13–15). In a previ-

∗To whom correspondence should be addressed.
E-mail: horiof@agr.nagoya-u.ac.jp
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A study conducted on humans, it was reported that AsA intravenously injected in patients with severe sepsis, reduced organ failure scores and serum levels of pro-inflammatory biomarkers (16).

The aim of this study was to verify the protective effects of AsA against LPS-induced sepsis in ODS rats, which are unable to synthesize AsA. The rats were divided into three groups and the dietary dose of AsA of each group was either high (3,000 mg/kg) (high-AsA), intermediate (300 mg/kg) (basal-AsA), or zero (AsA-free). We observed that septic death clearly decreased after feeding an AsA-containing diet compared to that after feeding an AsA-free diet. The higher the AsA dose in the diet, the greater was the survival rate of rats, showing that the protective effects of AsA were dose-dependent.

MATERIALS AND METHODS

Animals and diets. Male ODS (ODS/Shi Jcl-td/td) rats, 5 wk of age, were purchased from CLEA Japan, Inc. (Tokyo). They were housed in individual, wire screen-bottomed cages in the animal colony of Nagoya University and raised at temperature of 24 °C in a 12-h light cycle (lights on from 0800 h to 2000 h). The rats were allowed free access to water and a purified diet. The compositions of the AsA-free, basal-AsA, and high-AsA diets are shown in Table 1. The dietary addition of 300 mg of AsA/kg diet is sufficient for optimum growth and prevention of scurvy in ODS rats (3).

All the rats were fed the basal-AsA diet for 9 d before the commencement of the experiments, and then divided into three groups: AsA-deficient group, control group, and high-AsA group. The animal care and experimental procedures were approved by the Animal Research Committee of Nagoya University (approval number 2014021203) and were conducted according to the Regulations for Animal Experiments at Nagoya University.

Experiment 1. During the experimental period (8 d), ODS rats (initial body weight, 120–130 g) in the AsA-deficient group, control group, or high-AsA group were fed an AsA-free diet, a diet supplemented with 300 mg/kg AsA, or a diet supplemented with 3,000 mg/kg AsA, respectively. On day 8, 18 rats in each group were intraperitoneally injected with LPS (15 mg/kg body weight (BW) of rats, dissolved in saline, O55:B5 from Escherichia coli, Sigma L2880) at 1100 h. The survival rates of rats of all groups were calculated 48 h after the injection.

Experiment 2. We conducted a time-dependent study after the LPS injection. Rats in the AsA-deficient, control and high-AsA groups were fed their respective diets for 8 d. Then, rats in the three groups were intraperitoneally injected with LPS (15 mg/kg BW, dissolved in saline) at 1100 h of day 8, and killed by decapitation at 2, 6, or 10 h after the injection. Immediately before the LPS injection, that is, at 0 h too, rats in each group were also sacrificed. For each time point, six rats from each group were sacrificed. Blood was collected and serum was prepared by centrifugation at 1,500 × g for 10 min and stored at −20 °C until further use. Liver and spleen were removed, frozen immediately in liquid nitrogen, and stored at −80 °C.

Determination of ascorbic acid concentration in tissues. The liver and spleen were homogenized in ice-cold 50 g/L metaphosphoric acid and centrifuged for 15 min at 1,600 × g. The AsA concentration in the supernatant was measured by a modified dinitrophenylhydrazine method, in which 2,6-dichlorophenol-indophenol was used for oxidation of ascorbic acid (17).

Determination of serum levels of total bilirubin, AST, ALT, LDH, creatinine and BUN. The serum levels of total bilirubin, aspartate aminotransferase (AST), alanine transaminase (ALT), lactate dehydrogenase (LDH), creatinine and blood urea nitrogen (BUN) were measured by a commercial laboratory (SRL, Inc., Tokyo).

RNA preparation and gene expression analysis. Total RNA was extracted from whole blood using a commercial kit (RNeasy Protect Animal Blood, QUIAGEN GmbH, Hilden, Germany). cDNA was synthesized using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, Waltham, MA). Gene expressions were quantified by real-time PCR using an ABI 7300 real-time PCR system (Thermo Fisher Scientific) with the Thun-
Table 2. The primers for SYBR Green assay.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Genebank#</th>
<th>Sequence of oligonucleotides (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα</td>
<td>NM_012675</td>
<td>TCTTCTCATTTCCCTGCTGGTG</td>
</tr>
<tr>
<td>IL-1β</td>
<td>NM_031512</td>
<td>TTGGGAAACTCTCTCATCTGG</td>
</tr>
<tr>
<td>iNOS</td>
<td>NM_012611.3</td>
<td>GACCTGGTTCTTTGAGGCGTACAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AGATCCGGTCAGCAGCGTTGCTG</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ACCTTCCGACTTAGCACAGAA</td>
</tr>
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</table>

der PCR Mix or the Thunderbird SYBR qPCR Mix (Toyobo, Osaka). The primers used in the SYBR Green assay for TNFα, IL-1β and iNOS are shown in Table 2. TaqMan primers and probes were used to determine the level of IL-6 (TaqMan probe number, Rn.00561420_m1) and 18S rRNA (Eukaryotic 18S rRNA Endogenous Control) (TaqMan® Gene Expression Assays; Thermo Fisher Scientific). The level of each mRNA was normalized to that of the corresponding 18S rRNA.

**Determination of serum levels of cytokines.** The serum levels of TNFα and IL-1β were measured by enzyme-linked immunosorbent assay (ELISA) using a commercial assay kit (Quantikine ELISA kit; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. The minimum detection levels for TNFα and IL-1β were 25 pg/mL and 1 pg/mL serum, respectively.

**Determination of serum levels of nitrite and nitrate.** We measured serum levels of the sum of nitrite and nitrate originating from nitric oxide (NO). The serum was mixed with 10% trichloroacetic acid, and then centrifuged for 10 min at 7,000 ×g at 4°C. The total level of nitrite and nitrate in the supernatant, neutralized by 1 N NaOH, was measured with a commercial assay kit (NO2-/NO3- Assay Kit-C; Dojindo, Kumamoto, Japan).

**Statistical analysis.** The survival rates of rats of the control group and the high-AsA group were compared with that of the AsA-deficient group by using the Log-rank test. Differences with a p-value<0.05 were regarded as significant. The other results are expressed as mean±standard error, and the mean values were compared using the Tukey-Kramer method.

**RESULTS**

**Survival rates of ODS rats in the AsA-deficient, control, and high-AsA groups after the LPS injection (Experiment 1)**

After feeding the specific diet for 8d, the body weights of rats in the AsA-deficient, control, and high-AsA groups were 177.6 ± 1.9 g, 171.8 ± 3.8 g and 180.6 ± 3.2 g, respectively. Thus, there were no significant differences in body weight among these three groups. The rats of each group were intraperitoneally injected with LPS (15 mg/kg BW) on day 8. The survival rates of rats for each group, evaluated using the Kaplan-Meier method, after 48 h, are shown in Fig. 1. The survival rates, 48 h after LPS injection, were 5.5% (1/18), 39% (7/18) and 61% (11/18) in the AsA-deficient, control, and high-AsA groups respectively. The survival rates of rats in the control (p=0.0039) or the high-AsA (p=0.0007) group were significantly higher than that of rats in the AsA-deficient group.

**Body weights and tissue weights in the AsA-deficient, control, and high-AsA groups after the LPS injection (Experiment 2)**

Before LPS injection on day 8, there was no significant difference in the body weights (final body weight) among three groups (Table 3). During 10 h after the LPS injection, the relative liver weights decreased among the three groups, but the relative spleen weights increased. However, at 0 and 10 h, there were no significant differences in either liver or spleen weights among the AsA-deficient, control and high-AsA groups. Thus, the ingestion of AsA did not affect the weights of these tissues before or 10 h after the LPS injection.

**Tissue AsA concentrations in the AsA-deficient, control, and high-AsA groups after the LPS injection (Experiment 2)**

The AsA concentrations in the liver and spleen of the rats of the AsA-deficient group were significantly lower than those in the control group at all time-points (Fig. 2). These tissue AsA concentrations in the high-AsA group were significantly higher than those in the con-
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The tissue AsA concentrations at 6 and 10 h in both the control and high-AsA groups were approximately 60–80% of the AsA concentrations at 2 h in each group.

Serum levels of organ damage markers in the AsA-deficient, control, and high-AsA groups after the LPS injection (Experiment 2)

The serum levels of biomarkers of liver injury (total bilirubin, AST and ALT) and systemic organ injury (LDH) in the rats of the AsA-deficient group remarkably increased after the LPS injection, and remained higher than those in the rats of the control or high-AsA groups at 6 h after the LPS injection (Fig. 3A–D).

The serum levels of biomarkers of kidney injury (creatinine and BUN) in the rats of the high-AsA group were significantly lower than those in the rats of the AsA-deficient group at 10 h after the LPS injection (Fig. 3E and F).

Serum levels of inflammatory cytokines in the AsA-deficient, control, and high-AsA groups after the LPS injection (Experiment 2)

We measured serum levels of two inflammatory cytokines, that is, TNFα and IL-1β. The serum levels of TNFα before the LPS injection were below the minimum detection level, but they markedly rose at 2 h after the injection, and then again fell at 6 h (Fig. 4). After the LPS injection, the serum levels of TNFα in the rats of the control and high-AsA groups tended to be lower at 2 and 6 h, and were lower at 10 h than the corresponding values in the rats of the AsA-deficient group. At 6 and 10 h after LPS injection, the levels in rats of the control group were between those in the rats of the high-AsA and the AsA-deficient groups.

The serum IL-1β levels before the LPS injection were below the minimum detection level, but they increased until 6 h after the injection, and then again fell (Fig. 5). At 2 and 6 h, the IL-1β levels in the rats of the control and the high-AsA groups were lower than that of rats in the AsA-deficient group, but the difference was not significant. At 10 h after the injection, the IL-1β levels in the rats of the high-AsA group were significantly lower than those in the rats of the AsA-deficient group, and the levels in the rats of the control group showed intermediate values between those observed in the rats of the AsA-deficient and the high-AsA group.

Serum levels of nitrite and nitrate in the AsA-deficient, control, and high-AsA groups after the LPS injection (Experiment 2)

We also determined the sum of nitrite and nitrate levels in the serum. The elevation of these levels means the production of NO from the LPS treatment. The serum levels of nitrite and nitrate increased until 10 h after the LPS injection (Fig. 6). At 6 h, the levels in the rats of the high-AsA group were significantly lower than those in

Table 3. Initial body weight (day 0), final body weight (day 8) and organ weight in the AsA-deficient, control and high-AsA groups (Experiment 2).

<table>
<thead>
<tr>
<th></th>
<th>AsA-deficient</th>
<th>Control</th>
<th>High-AsA</th>
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<tr>
<td>Initial body weight (g)</td>
<td>127.5±1.8</td>
<td>127.2±2.4</td>
<td>127.3±1.5</td>
</tr>
<tr>
<td>Final body weight (g)</td>
<td>172.4±3.0</td>
<td>173.7±2.6</td>
<td>173.1±2.7</td>
</tr>
<tr>
<td>Tissue weight (g/100 g BW)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver 0 h</td>
<td>4.95±0.13</td>
<td>5.07±0.09</td>
<td>5.29±0.07</td>
</tr>
<tr>
<td>10 h</td>
<td>3.63±0.08</td>
<td>3.70±0.05</td>
<td>3.76±0.01</td>
</tr>
<tr>
<td>Spleen 0 h</td>
<td>0.290±0.009</td>
<td>0.279±0.006</td>
<td>0.298±0.005</td>
</tr>
<tr>
<td>10 h</td>
<td>0.321±0.009</td>
<td>0.319±0.006</td>
<td>0.335±0.005</td>
</tr>
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</table>

AsA, ascorbic acid; BW, body weight.

Values are mean±SE. n=6.
the rats of the AsA-deficient and the control groups.

The blood mRNA levels of inflammation-associated genes in the AsA-deficient, control, and high-AsA groups after the LPS injection (Experiment 2)

The mRNA levels of TNFα, IL-1β, IL-6 and iNOS in the total RNA isolated from whole blood of the rats were measured at 0, 2, and 6 h time-points. TNFα mRNA levels at 6 h after LPS injection in rats of the high-AsA group were significantly lower than those in rats of the AsA-deficient group (Fig. 7A). There was a decrease (not
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significant) in IL-1β mRNA levels at 2 and 6 h after LPS injection in rats of the high-AsA group compared to the levels in rats of the AsA-deficient group, and the levels in rats of the control group showed intermediate values compared to those in the rats of the other two groups (Fig. 7B). The IL-6 mRNA levels at 2 h after the LPS injection in rats of the high-AsA group were lower than those in rats of the AsA-deficient group, but the difference was not significant (Fig. 7C).

The iNOS mRNA levels at 6 h after LPS injection in rats of the high-AsA group were significantly lower than those in rats of the AsA-deficient group, and the levels in the control group showed intermediate values compared to the levels observed in the rats of the other two groups (Fig. 7D).

**DISCUSSION**

In this study, we investigated the protective effects of AsA against LPS-induced sepsis using ODS rats, which are unable to synthesize AsA. In ODS rats, the dietary AsA level of 300 mg/kg in the control group is sufficient to prevent the development of scurvy and was reported as the adequate dietary requirement of AsA (3). Feeding an AsA-free diet for 8 d to the rats in the AsA-deficient...
group did not cause growth retardation or any symptoms of scurvy, at all. Forty-eight hours after the injection of a lethal dose of LPS on day 8, the survival rate of the rats in the control group (39%) was significantly higher than that in the AsA-deficient group (5.5%). Notably, the survival rate of rats in the high-AsA group (61%) was much higher compared with that in the control group. These results demonstrated that the dietary intake of a dose of AsA (3,000 mg/kg diet) higher than the usual requirement imparted stronger protection against LPS-induced sepsis, and remarkably improved the survival rate compared with that of rats which were fed an AsA-free diet or a diet supplemented with just 300 mg/kg AsA.

To investigate the anti-inflammatory action of AsA in sepsis, we measured various parameters at 2, 6, and 10 h after the LPS injection. According to previous data in severely septic patients, multiple organ dysfunction syndrome (MODS) is often observed (12, 18). We measured the serum levels of organ injury markers to assess the effect of AsA on organ dysfunction. The serum levels of markers for hepatic injury, that is, total bilirubin, AST and ALT, were elevated and reached a maximum value at 6 h after the LPS injection in the rats of the AsA-deficient group; however, the levels of these markers were suppressed in the rats of the control and the high-AsA groups (Fig. 3A–C). Because these serum levels of the markers in the three groups at 6 h remained almost constant even 10 h after the LPS injection, it was inferred that AsA ingestion suppressed and delayed the development of hepatic injuries. The serum levels of the marker for systemic injury, LDH, showed similar trends among these three groups of rats as those observed for hepatic injury markers (Fig. 3D), which suggested that AsA suppressed organ dysfunctions induced by LPS not only in the liver but also in other organs. The serum levels of markers for renal injury, that is, creatinine and BUN, were elevated and reached a maximum at 10 h after the LPS injection, in the rats of the AsA-deficient group, and these elevations were suppressed in proportion to the AsA dose in the dietary intake of the rats of the control and high-AsA groups (Fig. 3E and F).

In the septic condition, inflammatory cytokines including TNFα and IL-1β, are produced by immune cells, resulting in SIRS. In this study, we measured the serum protein levels and the hematic gene expressions of TNFα and IL-1β after the LPS injection. Serum levels of IL-1β rose to a maximum value at 6 h after the LPS injection, and the elevated levels at each time point in the AsA-deficient group were higher than those in rats fed a diet supplemented with 300 or 3,000 mg AsA/kg (Fig. 5). Compared to the AsA-deficient group, hematic IL-1β mRNA levels at 2 and 6 h after LPS injection tended to be lowered by feeding AsA (Fig. 7B). Serum levels of TNFα were elevated to a maximum value at 2 h after the LPS injection, and the elevated levels at each time point in the AsA-deficient group tended to be lower in the control and high-AsA group (Fig. 4). However, the hematic TNFα mRNA was not elevated at 2 h after the LPS injection (Fig. 7A). It was speculated that the hematic TNFα mRNA level reached a maximum value between 0 and 2 h after the LPS injection, but we did not measure the level during that time period. The elevation of serum TNFα and IL-1β levels after LPS injection are caused by the stimulation of these gene expressions in hematic cells and several tissues. It is already known that the expressions of TNFα and IL-1β genes are induced by the activation of the transcriptional factor NF-κB during the LPS treatment (12). The phosphorylation of IκB, the core regulatory element for NF-κB function, is required for the activation of NF-κB. The phosphorylation of IκB is catalyzed by IκB kinase. Cárcamo et al. (19, 20) reported that vitamin C exerts the suppressive effects on the activation of NF-κB by inhibiting IκB kinase in several human cell lines. In this study, the intake of high doses of AsA might have suppressed the production of TNFα and IL-1β by inhibiting NF-κB activation by LPS in hematic cells and several tissues.

Hypotension is also a typical symptom of sepsis, leading to organ dysfunctions and death, and is caused by overproduction of NO, which inhibits oxygen transport (12, 18). We measured serum levels of nitrite and nitrate originating from NO (Fig. 6) and hematic iNOS mRNA levels (Fig. 7D). Six hours after the LPS injection, these levels were significantly lower in the rats of the high-AsA group compared with those in the AsA-deficient group. Although serum levels of nitrite and nitrate rose to a maximum value at 10 h after the LPS injection, these levels at 10 h among the three groups were not changed. These results suggested that the improvement of the survival rate in the high-AsA group was not caused by suppressing NO production.

In wild-type rats, which are able to synthesize AsA, it was shown that intravenous injection of AsA protected against the changes in hepatic vasoregulatory gene expressions during polymicrobial sepsis (15). Fisher et al. (13, 14) reported that in wild type mice, intraperitoneal injection of AsA after the LPS treatment improved the survival rate, prevented lung injury, and attenuated the expressions of proinflammatory chemokines in the lungs. Subsequently, Fisher et al. (21) also showed that intraperitoneal injection of AsA in AsA-deficient knockout mice (Gulo−/− mice), which are unable to synthesize AsA, suppressed injuries to the lung, kidney, and liver produced during sepsis caused by fecal stem solution. Recently, another group (22) reported that parenteral AsA attenuated the symptoms of sepsis and inhibited the immunosuppression of regulatory T cells in Gulo−/− mice with cecal ligation and puncture. As a noteworthy result, a human phase I trial showed that intravenous AsA infusion reduced the severe organ failures in septic patients (16). The present results demonstrated that the ingestion of a diet supplemented with a high dose of AsA as well as the parenteral administration of adequate amounts of AsA provided significant protection against septic damages.

In conclusion, we revealed the anti-inflammatory effects of dietary intake of AsA against LPS-induced sepsis in ODS rats, which included the improvement in survival rate and prevention of organ dysfunctions. In
particular, it was demonstrated for the first time that the intake of high doses of AsA in the high-AsA group showed the beneficial effects of AsA with more intensity as compared to the control group with only moderate doses of AsA. With therapeutic agents or treatments for sepsis still being sought, there is a possibility that dietary AsA will prove to be a protective and therapeutic agent against sepsis.

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Author contributions
N. Kawade, Y. Tokuda and S. Tsujino contributed equally to this work.

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